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## Modification of low density lipoprotein by advanced glycation end products contributes to the dyslipidemia of diabetes and renal insufficiency

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**ABSTRACT** Atherosclerosis develops rapidly in patients with diabetes or renal insufficiency. Plasma lipoprotein profiles are frequently abnormal in these conditions and reflect an elevation in the level of the apoprotein B (ApoB)-containing components very low density lipoprotein (VLDL) and low density lipoprotein (LDL). High levels of circulating advanced glycation end products (AGEs) also occur in diabetes and end-stage renal disease (ESRD). These products arise from glucose-derived Amadori products and include AGE-modified peptides (AGE-peptides) which result from the catabolism of AGE-modified tissue proteins. AGE-peptides have been shown to crosslink protein amino groups and to accumulate in plasma as a consequence of renal insufficiency. To address potential mechanisms for the dyslipidemia of diabetes and ESRD, we investigated the possibility that circulating AGEs react directly with plasma lipoproteins to prevent their recognition by tissue LDL receptors. AGE-specific ELISA showed a significantly increased level of AGE-modified LDL in the plasma of diabetic or ESRD patients compared with normal controls. AGE-LDL formed readily *in vitro* when native LDL was incubated with either synthetic AGE-peptides or AGE-peptides isolated directly from patient plasma. LDL which had been modified by AGE-peptides *in vitro* to the same level of modification as that present in the plasma of diabetics with renal insufficiency exhibited markedly impaired clearance kinetics when injected into transgenic mice expressing the human LDL receptor. These data indicate that AGE modification significantly impairs LDL-receptor-mediated clearance mechanisms and may contribute to elevated LDL levels in patients with diabetes or renal insufficiency. This hypothesis was further supported by the observation that the administration of the advanced glycation inhibitor aminoguanidine to diabetic patients decreased circulating LDL levels by 28%.

When compared with the general population, individuals with diabetes mellitus suffer a 3- to 4-fold increased risk for developing the complications of atherosclerosis and vascular insufficiency. Since diabetes has been estimated to afflict at least 10 million people in the United States alone, the contribution of diabetes to the overall mortality of heart disease and stroke is significant (1-3). A similar exacerbation of atherosclerotic vascular disease has been noted to occur independently of diabetes in patients with renal insufficiency. Individuals with renal failure in particular suffer from an accelerated vasculopathy that severely limits long-term survival on hemodialysis therapy (4).

Diabetic and renally impaired patients with or without diabetes have been found to exhibit high circulating levels of protein-bound advanced glycation end products (AGEs) (5-

7). Advanced glycation is initiated by the nonenzymatic attachment of glucose to amino groups, forming covalently bound but slowly reversible Amadori products. Rearrangement reactions then occur to produce a chemically related group of moieties, termed AGEs, that remain irreversibly bound to proteins. AGEs are highly reactive and over time continue to react with nearby amino groups to produce both intra- and intermolecular crosslinks (7-10). Although circulating AGEs form in part by the *in situ* reaction of glucose with serum proteins, a large portion of these products enter the plasma compartment as AGE-modified peptides (AGE-peptides) via catabolism of AGE-modified tissue proteins. Thus, extremely high concentrations of circulating AGE-peptides occur even under nonhyperglycemic conditions if plasma filtration is impaired by renal failure. AGE-peptide levels are measurably elevated in individuals with only a mild to moderate degree of renal insufficiency, and there is a direct correlation between serum AGE-peptide levels and renal function as assessed by creatinine clearance (5).

AGEs have been shown to account in part for the increase in collagen crosslinking that accompanies connective tissue aging and which occurs at an accelerated rate in diabetes (11-13). Protein-bound AGEs also are recognized and taken up by AGE-specific receptors that have been identified to be present on diverse cell types (14). Occupancy of these receptors by AGE-modified proteins promotes local cytokine release, contributing to normal tissue remodeling processes (15). AGEs also interact with endothelial cell AGE receptors to increase vascular permeability and up-regulate the synthesis of a variety of products, including tissue factor, a cell surface procoagulant (16). Subendothelial AGEs have been found to chemically inactivate nitric oxide (endothelial-derived relaxing factor) and appear to play a role in age- and diabetes-related abnormalities in vascular responsiveness (17, 18). Thus, a variety of observations suggest that AGEs contribute significantly to the multiorgan complications of both diabetes mellitus and normal aging.

Patients with diabetes or renal insufficiency frequently suffer from a complex dyslipidemia which is characterized in part by defective lipoprotein uptake and metabolism (19-22). We recently reported the presence of an AGE-modified form of low density lipoprotein (LDL) which circulates in higher amounts in diabetic than in nondiabetic patient plasma (23). These AGE modifications are present on both the apoprotein B (ApoB) and the phospholipid components of LDL and contribute directly to the oxidative modification of the LDL

Abbreviations: AGE, advanced glycation end product; AGE-peptide, AGE-modified peptide; ApoB, apoprotein B; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; ESRD, end-stage renal disease; HDL, high density lipoprotein; LDL, low density lipoprotein; U, units; VLDL, very low density lipoprotein.

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particle. In the present study, we report that AGE-modified LDL forms rapidly by the direct reaction of native LDL with circulating reactive AGE-peptides. LDL modified by AGE-peptides exhibited markedly impaired clearance kinetics when injected into transgenic mice expressing the human LDL receptor. Furthermore, administration of the advanced glycosylation inhibitor aminoguanidine to diabetic patients was found to decrease circulating LDL levels by 28% over a 4-week period.

## MATERIALS AND METHODS

**AGE-LDL Measurement.** Plasma LDL (1.025–1.063 g/ml) was isolated from patient plasma by sequential ultracentrifugation (24). The ApoB and lipid components were separated by extracting LDL solutions with chloroform/methanol (1:1, vol/vol) and pelleting the denatured apoproteins by microcentrifugation. After washing in distilled H<sub>2</sub>O, 2–4 mg of apoprotein was incubated with 1 mg of proteinase K for 18 hr at 37°C. The ApoB-AGE content of the digested material was measured by competitive AGE ELISA as described (6, 23). Lipid-AGEs were measured after drying the lipid-soluble material under a stream of nitrogen, redissolving it in methanol, and assaying triplicate 0.1-ml aliquots by direct AGE ELISA (23). Data for each LDL group are expressed as the mean  $\pm$  SEM of AGE units (U) calculated relative to a synthetic AGE-albumin standard (6).

**Preparation of AGE-Peptides and AGE-LDL.** Synthetic AGE-peptides were prepared by proteolysis of AGE-modified albumin (AGE-BSA) (6). Briefly, 5 ml of AGE-BSA (30 mg/ml, in phosphate-buffered saline (PBS; 145 mM NaCl/10 mM sodium phosphate, pH 7.4)) was incubated with proteinase K (5 mg) overnight at 37°C. Peptides then were isolated by centrifugation of digested material through a Centrprep-10 membrane (10-kDa molecular mass cutoff) (Amicon). Human plasma AGE-peptides were prepared similarly by centrifuging freshly isolated whole plasma through Centrprep-10 membranes. The AGE specific activity of each peptide fraction was determined by ELISA prior to incubation with LDL. For AGE-LDL preparation, purified native LDL (3.3 mg/ml) was incubated at 37°C for 1–14 days in PBS containing 0.01% EDTA and 0.2 M glucose, synthetic AGE-peptides (45 mg of protein per ml at an AGE specific activity of 100 U/mg of protein), or AGE-peptides isolated from diabetic/end-stage renal disease (ESRD) plasma (80 mg of plasma protein equivalent per ml at an AGE specific activity of 90 U/mg). Aliquots of LDL then were dialyzed against PBS and subjected to ELISA analysis for ApoB- and lipid-AGEs.

**Transgenic Mouse Studies.** Mice transgenic for the human LDL receptor under control of the mouse metallothionein I

promoter were used for these studies (25). AGE-LDL was prepared by incubating native LDL with synthetic AGE-peptides *in vitro* (described above) so as to achieve a level of AGE modification comparable to that observed in diabetic ESRD patients *in vivo* ( $\approx 80$  U of AGE per mg of ApoB). Purified native LDL was incubated with synthetic AGE-peptides for 17 hr at 37°C in PBS containing EDTA (1 mM) and butylated hydroxytoluene (BHT; 20  $\mu$ M). The absence of oxidative changes was ensured by assaying AGE-LDL for reactivity with thiobarbituric acid (26). Seventeen hours before use, mice were injected intraperitoneally with cadmium sulfate at 0.8 mg/kg of body weight. On the day of study, anesthesia consisting of sodium pentobarbital (25 mg) was administered intraperitoneally prior to injection of each tracer. Two and one-half microcuries (1  $\mu$ Ci = 37 kBq) of radioiodinated LDL (<sup>125</sup>I for AGE-LDL and <sup>131</sup>I for native LDL) (27) was administered intravenously into tail veins, and blood samples (25  $\mu$ l) were collected at 0, 1, 5, 15, 45, 150, and 210 min later. Radioactivities of 5- $\mu$ l aliquots of plasma samples were measured in 20-min counts. The fraction of each injected dose of AGE-LDL or native LDL which remained in plasma was obtained by dividing the <sup>125</sup>I or <sup>131</sup>I dpm at each time point by the corresponding dpm at 1 min after injection. The ratio of AGE-LDL to native LDL was calculated from the fraction of injected AGE-LDL remaining in plasma divided by the corresponding fraction of injected native LDL, and the ratio was averaged for all injected mice at each time point. Five mice were used for each pair of tracers in the control nontransgenic group and six mice were used in the transgenic group.

**Patient Studies.** Informed consent was obtained from 18 diabetic patients, who received aminoguanidine daily for 28 days. Twelve patients had type I diabetes and 6 patients had type II diabetes. The mean age was 45.7 years, and the mean duration of diabetes was 20.4 years. Nine patients exhibited various degrees of renal impairment (six patients had a creatinine clearance of 30–80 ml/min and three patients had a creatinine clearance of <30 ml/min). Aminoguanidine was administered orally at an average dose of 1200 mg/day. Dosage was adjusted as necessary in selected patients to achieve a mean (trough) plasma level of 10  $\mu$ g/ml. A 5-ml sample of whole blood was collected into heparinized tubes at the initiation and termination of treatment. HbA<sub>1c</sub> (hemoglobin A N-terminally glycosylated on the  $\beta$  chain) was measured by HPLC (28) and Hb-AGE was determined by ELISA with reference to an AGE-BSA standard (29). Lipoprotein profiles were determined at the Institute of Human Nutrition, University of Texas, Southwestern Medical School, following a previously published method (30).

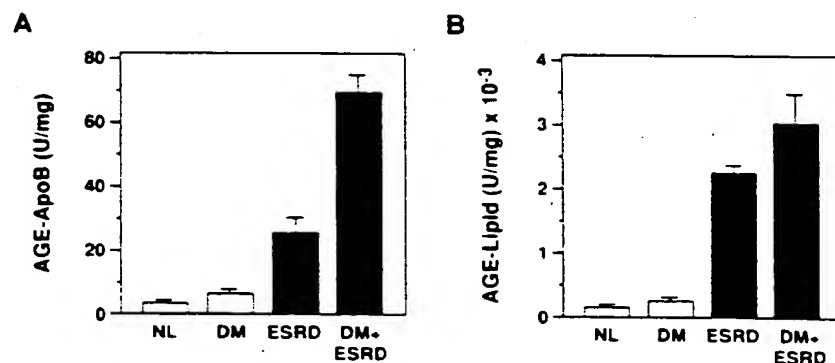


FIG. 1. LDL-associated AGEs (AGE-LDL) measured in 17 normal controls (NL), 22 diabetic patients with normal renal function (DM), 8 patients with ESRD but without diabetes (ESRD), and 12 patients with both diabetes and ESRD (DM + ESRD). (A) AGE-ApoB analysis. (B) AGE-phospholipid analysis. Bars represent mean  $\pm$  SEM.  $P < 0.001$  (Student's *t* test) for each patient group versus the normal control group.

## RESULTS

Plasma AGE-LDL levels were measured in 59 patients with normal renal function (17 nondiabetic, 22 diabetic), 8 patients with ESRD but without diabetes, and 12 patients with both diabetes and ESRD (Fig. 1). LDL was isolated and fractionated into its apoprotein (ApoB) and lipid components, and AGE modification was quantitated by AGE-specific ELISA. There was a significant elevation in the levels of ApoB-AGE and lipid-AGE in LDL when each of the patient groups was compared to the control (nondiabetic/normal renal function) group. Normal controls showed a mean AGE of  $2.8 \pm 0.12$  U/mg of ApoB. ApoB-AGE levels were elevated 1.4-fold in the diabetic group ( $4.0 \pm 0.3$  U/mg of protein), 9-fold in the nondiabetic/ESRD group ( $27.6 \pm 5.8$  U/mg of protein), and 24-fold in the diabetic/ESRD group ( $70.6 \pm 6.3$  U/mg of protein). The mean level of lipid-AGEs in the LDL of control subjects was found to be  $115 \pm 6$  U/mg of lipid. Lipid-AGE levels were elevated 2-fold in the diabetic group ( $234 \pm 21$  U/mg of lipid), 17-fold in the nondiabetic/ESRD group ( $2091 \pm 204$  U/mg of lipid), and 27-fold in the diabetic/ESRD group ( $3266 \pm 650$  U/mg of lipid). The markedly increased level of AGE modification of lipid compared with apoprotein is consistent with *in vitro* studies showing that advanced gly-

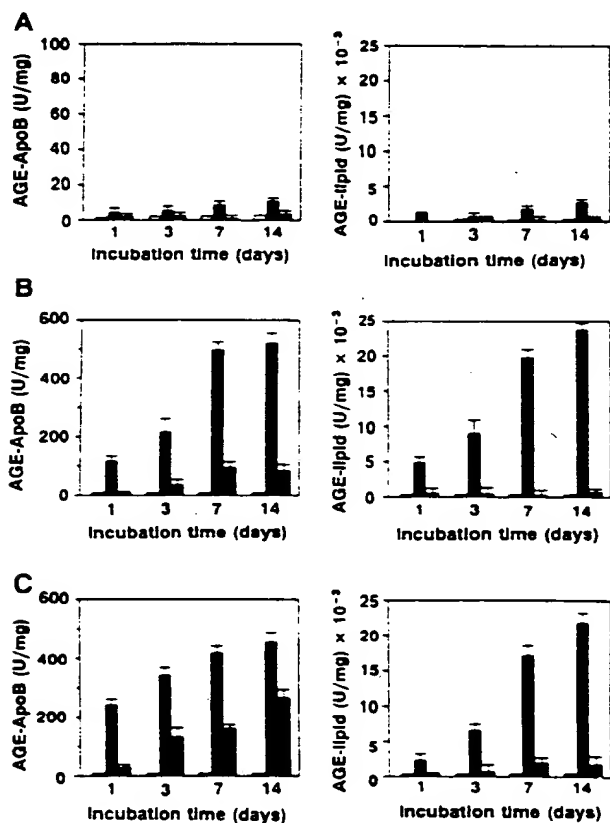


FIG. 2. Formation of AGE-LDL *in vitro*. Purified native LDL (3.3 mg/ml) was incubated at 37°C in PBS containing glucose (0.2 M) (A), synthetic AGE-peptides (45 mg of protein per ml at an AGE specific activity of 100 U/mg of protein) (B), or AGE-peptides isolated from diabetic/ESRD plasma (80 mg of plasma protein equivalent per ml at an AGE specific activity of 90 U/mg) (C). Aliquots of LDL were removed at intervals and dialyzed to remove glucose or unbound AGEs, and the ApoB and lipid components were subjected to AGE ELISA. Empty bars, control incubations with LDL alone; solid bars, LDL incubated with glucose or AGE-peptides; shaded bars, LDL incubated with glucose or AGE-peptides, plus aminoguanidine (300 mM). Bars represent mean  $\pm$  SEM.

cation proceeds rapidly within the apolar microenvironment of lipid subphases (23).

To address potential mechanisms for the increased level of AGE-modified LDL in diabetic and ESRD plasma, native

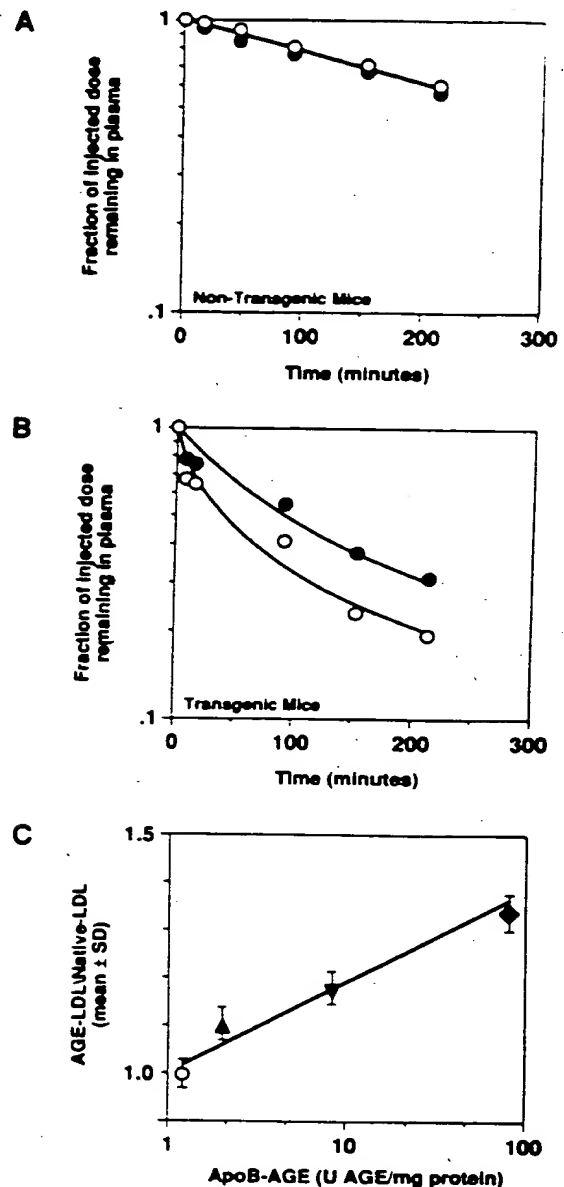


FIG. 3. Plasma clearance of native and AGE-LDL in control nontransgenic mice and transgenic mice expressing the human LDL receptor. (A) Control (nontransgenic) mice injected with native LDL (○) or AGE-LDL (●). (B) Mice transgenic for the human LDL receptor injected with native LDL (○) or AGE-LDL (●). The ratio of AGE-LDL to native LDL was calculated and averaged for all mice at each time point. The mean clearance ratio of AGE-LDL to native LDL in the transgenic mice was determined to be  $1.35 \pm 0.03$  ( $P < 0.001$  compared with a ratio of 1 by one-way ANOVA). (C) Relationship between the extent of ApoB-AGEs and the AGE-LDL/native-LDL clearance ratio. ApoB-AGEs were measured by ELISA in four different preparations of LDL that were subjected to plasma clearance studies. ○, Control (native) LDL (1.2 U of AGE per mg of ApoB); ▲, LDL modified with synthetic AGE-peptides in the presence of 800 mM aminoguanidine, yielding 2.0 U of AGE per mg of ApoB; ▼, LDL modified with synthetic AGE-peptide in the presence of 400 mM aminoguanidine, yielding 8.0 U of AGE per mg of ApoB; ◆, LDL modified with AGE-peptide alone (80 U of AGE per mg of ApoB).

Table 1. Biochemical analysis of blood specimens obtained from diabetic patients who received aminoguanidine ( $n = 18$ ) or placebo control ( $n = 8$ ) for 28 days

Treatment	% of baseline value						
	Cholesterol	Triglyceride	VLDL	LDL	HDL	Hb-AGE	HbA <sub>1c</sub>
Aminoguanidine	81.3 $\pm$ 7.2*	81.0 $\pm$ 6.2*	68.5 $\pm$ 28.7	71.9 $\pm$ 9.9*	104.7 $\pm$ 10.9	72.7 $\pm$ 7.5†	89.7 $\pm$ 4.2
Placebo	97.4 $\pm$ 5.4	89.8 $\pm$ 5.8	96.4 $\pm$ 7.1	100.7 $\pm$ 11.2	96.7 $\pm$ 16.4	90.8 $\pm$ 6.7	100.0 $\pm$ 4.5

Values are expressed as percent (mean  $\pm$  SEM) of baseline value for each patient group [(day 28 value/day 0 value)  $\times$  100].  $P$  values compared with baseline, calculated by paired Student's  $t$  test, are as follows: \*,  $P < 0.04$ ; †,  $P < 0.02$ ; ‡,  $P < 0.05$ ; §,  $P < 0.025$ ; unmarked values are not significantly ( $P > 0.05$ ) different from 100.

LDL was incubated with glucose or AGE-peptides which were either synthesized *in vitro* or isolated from the plasma of patients with diabetes and ESRD. As expected, LDL incubated with glucose showed a gradual time-dependent increase in the content of both ApoB-AGEs and lipid-AGEs (Fig. 2). Of significance, however, the formation of ApoB-AGEs and lipid-AGEs proceeded much more rapidly in the presence of reactive AGE-peptides. This enhanced rate of AGE-LDL formation occurred both with synthetic AGE-peptides and with AGE-peptides isolated from patient plasma. These data affirm the inherent crosslinking activity of circulating AGEs and point to the potential importance of these plasma products in producing rapid chemical modification of LDL *in vivo*. Given the relatively short circulating half-life of LDL under normal circumstances [36–48 hr (31, 32)], it is likely that reactive plasma AGEs contribute significantly to the formation of AGE-modified LDL in diabetic and ESRD patients. It also is noteworthy that the time-dependent formation of ApoB-AGEs and lipid-AGEs was inhibited by addition of the advanced glycation inhibitor aminoguanidine (33) (Fig. 2).

Chemical modification of basic residues within the LDL-receptor-binding domain of ApoB has been shown to interfere with the ability of LDL to undergo receptor-mediated uptake and degradation (34, 35). Advanced glycation similarly modifies the lysine and arginine residues of proteins (9, 10), and it was reasoned that LDL modified irreversibly by AGEs might exhibit markedly delayed clearance kinetics *in vivo*. Although Amadori products have been identified to be present on human LDL, these early glycation products do not affect LDL clearance unless the reversible ketoamine linkage is first stabilized by chemical reduction with borohydride (36).

The plasma clearance of AGE-modified LDL was examined in transgenic mice expressing the human LDL receptor, an animal model which has been shown to be useful in evaluating the clearance of abnormal human LDL particles (25). AGE-LDL was prepared by incubating native LDL with synthetic AGE-peptides for 17 hr at 37°C. These reaction conditions were selected to achieve a level of AGE modification comparable to that observed in diabetic/ESRD patients *in vivo* ( $\approx 80$  U of AGE per mg of ApoB; Fig. 1). The antioxidants BHT and EDTA were included during the preparation of AGE-LDL to minimize effects that potentially could be attributed to oxidative modification (37). AGE-modified and native LDL then were radioiodinated with  $^{125}\text{I}$  and  $^{131}\text{I}$ , respectively, and injected by tail vein into groups of five or six mice. Plasma aliquots were obtained at intervals after injection and analyzed for radioactivity. The fraction of AGE-LDL or LDL which remained in the plasma was calculated by dividing the respective  $^{125}\text{I}$  or  $^{131}\text{I}$  dpm at each time point by the dpm at 1 min after injection. As shown in Fig. 3, AGE-LDL exhibited markedly delayed clearance kinetics compared with native LDL. The specificity of the AGE effect for LDL-receptor-mediated uptake was validated by performing parallel studies in nontransgenic mice. In these mice, which lack the human LDL receptor, no difference was observed in the relative clearance rates of AGE-modified

versus native LDL. In further experiments, native LDL was incubated with reactive synthetic AGEs in the presence of the advanced glycation inhibitor aminoguanidine. As expected, there was a lower level of AGE modification under these reaction conditions, and injection of these preparations of AGE-LDL into human LDL-receptor transgenic mice showed an improvement in plasma clearance kinetics (Fig. 3C). Overall, there was a significant relationship between the extent of ApoB-advanced glycation and impaired plasma clearance of LDL *in vivo*.

To begin to assess the contribution of advanced glycation to altered LDL clearance kinetics in human subjects, plasma lipoprotein profiles were examined in diabetic patients with elevated serum AGE-peptide levels who were enrolled in a 28-day double-blind placebo-controlled trial of aminoguanidine (29). Eighteen patients received aminoguanidine at an average daily dose of 1200 mg and eight patients received a placebo. Blood samples were obtained at the initiation and termination of treatment and analyzed for total cholesterol, triglycerides, very low density lipoprotein (VLDL)-cholesterol, LDL-cholesterol, high density lipoprotein (HDL)-cholesterol, Hb-AGE, and HbA<sub>1c</sub> (Table 1). The efficacy of aminoguanidine as an inhibitor of advanced glycation in these subjects was verified in this trial by the observation that circulating Hb-AGE levels decreased by almost 28% in the aminoguanidine-treated group (29). Of significance, aminoguanidine therapy also was associated with a 19% decrease in total cholesterol, a 19% decrease in triglycerides, and a 28% decrease in LDL-cholesterol. There was a trend toward a decrease in VLDL-cholesterol as well, but this difference did not reach statistical significance. The reduction in plasma lipids could not be attributed to a simple improvement in metabolic control, since the mean HbA<sub>1c</sub> value was not significantly affected during the trial by aminoguanidine treatment. No significant differences in the HbA<sub>1c</sub>, Hb-AGE, lipid, or lipoprotein profiles could be detected in the placebo-treated group over the 28-day study period. Although it also would have been informative to have measured AGE modification of LDL before and after aminoguanidine therapy, insufficient quantities of plasma were available from this study to perform this analysis.

## DISCUSSION

Among the pathological processes believed to be central in the development of atherosclerosis are biochemical modifications that affect the functional integrity of the LDL particle. These modifications may act to decrease plasma LDL clearance, increase its residence time within the subendothelium, or divert the normal pathway of LDL metabolism from tissues to vascular wall macrophages, leading to foam cell formation (37, 38). AGEs form ubiquitously on the amino groups of tissue and plasma proteins, and the absolute level of AGE modification is a function of both ambient blood glucose levels and the half-life of the protein substrate. The observation that measurable quantities of AGEs occur on short-lived plasma proteins such as LDL prompted the consideration that these modifications may arise from reactive, circulating AGE-peptides instead of glucose. This hy-

pothesis was further supported by the finding that AGE-LDL levels are increased in patients with renal insufficiency independently of diabetes. AGE-LDL forms in these patients by the reaction of native LDL with circulating AGE-peptides which accumulate in the plasma during renal compromise.

In contrast to native LDL, AGE-LDL was found to exhibit markedly delayed clearance kinetics when injected into transgenic mice expressing the human LDL receptor. Plasma clearance was found to be related directly to the level of AGE modification of LDL, and clearance kinetics were improved by preparing AGE-LDL in the presence of the advanced glycation inhibitor aminoguanidine.

The finding of a specific decrease in circulating LDL levels in aminoguanidine-treated diabetic patients provides further support for the hypothesis that advanced glycation of LDL contributes significantly to impaired LDL clearance *in vivo*. These data are consistent with the notion that AGEs form at sites located in, or near, the LDL-receptor-binding domain, thereby interfering with the uptake of LDL by tissue LDL receptors. AGEs also have been shown to form on the phospholipid components of LDL, and the initiation of oxidative modification by phospholipid-linked AGEs may contribute further to the chemical modification of LDL induced by advanced glycation reactions (23).

In summary, the elevated plasma level of AGE-LDL in diabetic and renally impaired patient groups, the rapid chemical modification of LDL by reactive plasma AGEs *in vitro*, and the impaired clearance kinetics of AGE-LDL in human LDL-receptor transgenic mice point to the important role of advanced glycation of LDL in the dyslipidemia of diabetes and ESRD. Impairment in the normal receptor-mediated clearance of LDL is likely to act in concert with abnormalities in lipoprotein production, increases in lipoprotein oxidation and vascular wall lipoprotein trapping, and alterations in endothelial cell function (37, 38) to produce the rapidly progressive vasculopathy of diabetes or ESRD. Lower levels of AGE-modified LDL occur in nondiabetic/non-renally impaired individuals, and it is worthwhile to consider that over a time period of many years, advanced glycation also may contribute to the age-related development of atherosclerosis in the general population.

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